MOLECULAR CHARACTERIZATION OF TWO COMPONENTS OF THE IP_3 SIGNALLING PATHWAY IN THE LOBSTER OLFACTORY ORGAN

By

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To Peter and Joan Munger, for everything.

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Abstract of Dissertation Presented to the Graduate School
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MOLECULAR CHARACTERIZATION OF TWO COMPONENTS OF THE IP_3 SIGNALLING PATHWAY IN THE LOBSTER OLFACTORY ORGAN

By

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Chairman: Barry W. Ache
Major Department: Neuroscience

Olfactory transduction is a complex biochemical process that transforms odor signals from the environment into neural signals that can be interpreted by the brain, triggering appropriate behavioral and physiological responses in the organism. Inositol 1,4,5-trisphosphate (IP_3) has been implicated, in a variety of species, as a second messenger involved in olfactory transduction, though its precise role remains controversial. IP_3 has been proposed by some to be either a modulatory factor or a necessary cofactor for the better characterized transduction pathway mediated by adenosine 3',5'-cyclic monophosphate (cAMP); others believe it to have a role equivalent, but not identical, to cAMP. A key to understanding the funtional intricacies of the cAMP pathway was the molecular isolation and characterization of components of this signalling cascade in olfactory receptor neurons (ORNs). Unfortunately, few

components of the IP_3 pathway of ORNs have been characterized. In the spiny lobster, *Panulirus argus*, electrophysiological studies have implicated an IP_3-gated channel, the IP_3 receptor (IP_3R), as the target of IP_3 generated by excitatory odor-stimulation. A Gq protein α-subunit, a member of the family of heterotrimeric GTP-binding proteins, has also been implicated in the excitatory odor response, serving to link the activated odor receptor with the enzyme that generates IP_3. This dissertation describes the molecular cloning of cDNAs for two components of an IP_3 signalling cascade from the olfactory organ (nose) of the spiny lobster: an IP_3 receptor and a Gq protein. These two proteins, both implicated in the excitatory odor response in lobsters, share many structural characteristics with those isolated from other species and tissues. The IP_3 receptor cDNA encodes a protein that is about 50% identical to those isolated from *Drosophila* and several vertebrates. The Gq protein shows an even similarity to other Gq proteins (70-80%). The messenger RNAs for these two molecules are both expressed in the olfactory organ, making them candidates for roles in olfactory transduction. The isolation and characterization of these two cDNAs is a critical step in the understanding of both the role of IP_3 in olfactory signalling and the complexity of odor transduction in all animals.

CHAPTER 1
INTRODUCTION

Olfactory Processing

Most odors are complex mixtures of compounds, and it is the role of the olfactory system to transduce these chemical stimuli into neural signals that reflect the complex nature of the odor. The olfactory system does this by doing more than just assigning each odor component a neural signature, reflected as a change in membrane potential. The system also processes the signals to reflect such information as concentration and mixture composition. This processing could take place at several possible levels. In the extracellular environment, access of odor molecules to olfactory receptor binding sites on the surface of olfactory receptor neurons could be regulated by odorant binding proteins or enzymes that degrade odor molecules, or there could be competition between odor molecules for olfactory receptor binding sites. In olfactory receptor neurons that contain multiple transduction cascades, the activation of olfactory receptors of different specificity could activate more than one transduction pathway, providing ample targets for biochemical cross-talk that might modulate the function of either cascade. The best characterized site of olfactory processing is at the level of synaptic processing, beginning at the point of contact between olfactory receptor neurons contact second order neurons in the olfactory bulb (vertebrates) or lobe (invertebrates) and continuing in higher neural centers; here, the activity of numerous olfactory receptor neurons is shaped by the integration of activity across the population. Although each of these levels is a potentially critical step in the coding and processing of odor information, this dissertation will concentrate on some of the mechanisms of olfactory transduction.

Through the better understanding of these mechanisms in cells that express multiple transduction cascades, it may be possible to decipher how individual receptor neurons could begin to process multiple odor stimuli at the level of the single receptor cell.

Multiple Transduction Pathways in Olfaction

Olfactory transduction is thought to occur through standard G protein-mediated second messenger systems (reviews: Breer and Boekhoff, 1992; Ache and Zhainazarov, 1995; Restrepo *et al.*, 1996; but see Vodyanoy and Murphy, 1983; Labarca *et al.*, 1988). In such a system, a receptor is coupled to second messenger-metabolizing enzymes (e.g. adenylyl cyclase, which synthesizes cAMP from ATP) by a heterotrimeric GTP-binding protein (G protein). Evidence in rat (Breer *et al.*, 1990; Boekhoff *et al.*, 1990; Ronnett *et al.*, 1993), amphibian (Dionne, 1992; Bacigalupo *et al.*, 1993; Lowe *et al.*, 1989; Lischka *et al.*, 1995), catfish (Ivanova and Caprio, 1992; Miyamoto *et al.*, 1992; Restrepo *et al.*, 1990; Bruch and Teeter; 1990), lobster (Michel and Ache, 1992; Fadool and Ache, 1992; Boekhoff *et al.*, 1994) and squid (Lucero *et al.*, 1992; Lucero and Piper, 1994) points to multiple olfactory transduction mechanisms, and implicates adenosine 3',5'-cyclic monophosphate (cAMP) and inositol 1,4,5-trisphosphate (IP_3) as olfactory second messengers (review: Ache, 1994). However, the functional consequences of cAMP and IP_3 vary between species. For example, while cAMP and IP_3 both depolarize ORNs in the catfish (Ivanova and Caprio, 1992), the two messengers mediate opposing conductances in the lobster (Fadool and Ache, 1992; Michel and Ache, 1992). cAMP and IP_3 both appear to act by directly gating ion channels in the plasma membrane (Nakamura and Gold, 1987; Zufall *et al.*, 1994; Fadool and Ache, 1992; Hatt and Ache, 1994; Lischka *et al.*, 1994), though some have challenged the findings of a plasma-membrane IP_3-gated channel (Brunet *et al.*, 1996; Nakamura *et al.*, 1996). There is now growing evidence for a multitude of modulatory and amplifying factors that may act on

one or both of these two pathways, including the gaseous messengers nitric oxide and carbon monoxide (Lischka and Schild, 1993; Leinders-Zufall *et al.*, 1995; Zufall, 1996; Broillet and Firestein, 1996), Ca^{2+} (e.g. Anholt and Rivers, 1990; Kleene, 1993; Lowe and Gold; 1993; Kurahashi and Yau, 1994) and cGMP (Breer *et al.*, 1992) (Figure 1).

The advent of molecular biology has afforded the opportunity to identify many of the molecules that compose these olfactory transduction pathways, permitting better characterization of their functions and their distributions. Most of the major components of the olfactory cAMP pathway have been cloned from mammals: a type III adenylyl cyclase (Bakalyar and Reed, 1990); a G protein α-subunit, G_{olf} (Jones and Reed, 1989); and two subunits of the cAMP-gated channel (Dhallan *et al.*, 1990; Bradley *et al.*, 1994; Liman and Buck, 1994). The IP_3 pathway is not nearly as well characterized, with only a catfish phospholipase C (Abogadie *et al.*, 1995a), a lobster IP_3-gated channel (Munger *et al.*, 1995; Munger *et al.*, 1997; Chapter 2), two lobster Gq proteins (Munger *et al.*, 1997; Chapter 3; Xu *et al.*, 1997) and a *Drosophila* Gq protein (Talluri *et al.*, 1995) having been cloned.

The lobster as an olfactory model system

One advantage of the spiny lobster, *Panulirus argus*, as a model system for the study of olfactory transduction is the extensive characterization of behaviorally-relevant odors. Many of these odors are small hydrophilic compounds, such as amino acids, quatenary ammonium compounds, nucleotides, and amines (review: Carr *et al.*, 1987; Ache and Carr, 1989), present in the flesh of the animal's typical prey. Electrophysiological and biochemical of the ORNs to many of these odors, as well as behavioral responses of the animal, have been well characterized .

The olfactory organ, or the "nose," of the spiny lobster is the two lateral filaments of paired, bifurcated antennules (Figure 2). The somata of the olfactory receptor neurons

Figure 1. Proposed pathways in olfactory transduction. **(A)** A vertebrate olfactory receptor neuron (ORN). Odor transduction takes place in the dendritic cilia, while the axon projects to the CNS, where it makes its first synapse. Invertebrate ORNs have a similar structure (see Figure 2). **(B)** These biochemical pathways have been proposed in a variety of organisms to mediate olfactory transduction. The better characterized pathway involves the activation of adenylyl cyclase (AC) after odor activation of a receptor (R), via a G protein (G). The AC generates cAMP, which then directly gates a membrane channel. The second, parallel pathway, involves separate receptors, G protein, and the metabolizing enzyme phospholipase C (PLC), which generates the messengers IP_3 (or $InsP_3$) and diacylglycerol. IP_3 ($InsP_3$) can also directly gate its own membrane channel. Several potential modulatory and amplification pathways are also proposed, including the activation of protein kinase A (PKA), protein kinase C (PKC) and a G protein-coupled receptor kinase (GRK). Ca^{2+}, entering the cell through either second messenger-gated channel, can act both through calmodulin (CAM), such as by acting on phosphodiesterase (PDE), and by directly gating other channels. Finally, IP_3 ($InsP_3$) can be phosphorylated by a 3-kinase into $InsP_4$, which may also gate a channel. From Ache and Zhainazarov, 1995, with permission.

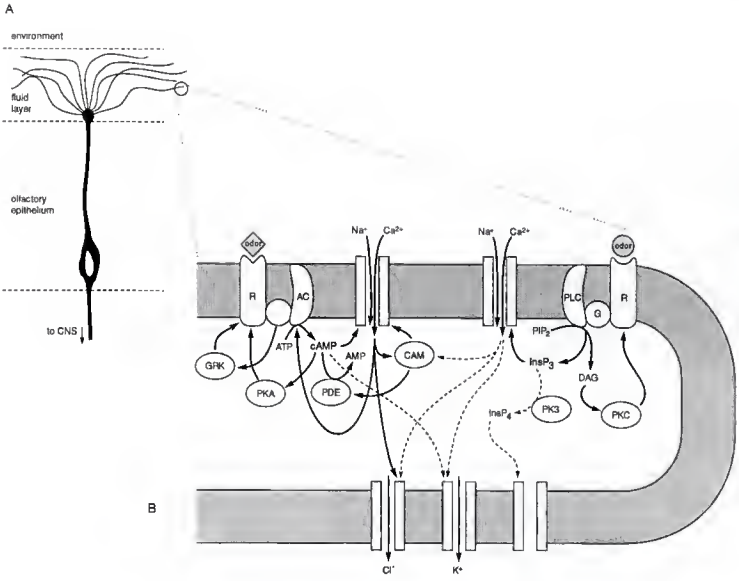
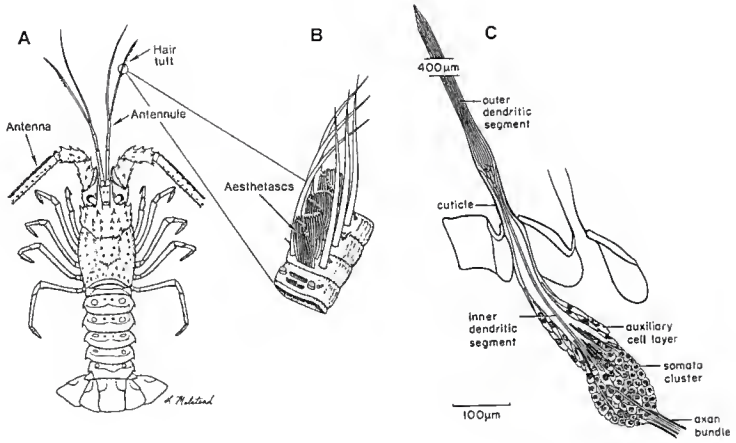

A
environment
fluid layer
olfactory epithelium
to CNS
B
odor
R
AC
ATP
cAMP
AMP
GPK
PKA
PDE
CAM
Na+
Ca2+
Na+
Ca2+
PLC
G
R
odor
PIP2
InsP3
DAG
InsP4
PK3
PKC
Cl-
K+

Figure 2. **(A)** The lateral filament of the antennule of *P. argus* is the olfactory organ. The expanded view at right **(B)** shows the aesthetasc sensilla between two rows of guard hairs. The sensilla contain the dendrites of the ORNs, while the ORN somata lie within the antennular lumen. **(C)** A cross section of a single aesthetasc sensillum shows the cluster of somata surrounded by auxiliary cells, projecting their dendrites into the shaft of the aesthetasc. The axons of the ORNs project to the olfactory lobe of the brain, where they make their first synapse.


A
Hair tuft
Antennule
Antenna
B
Aesthetascs
C
400µm
outer dendritic segment
cuticle
inner dendritic segment
auxiliary cell layer
somata cluster
axon bundle
100µm

(ORNs) are clustered like bunches of grapes on the ventral aspect of the antennular lumen. The ciliated dendrites of these bipolar neurons extend into porous cuticular sensilla (aesthetascs), while the axons project to the CNS. Each sensillum has a cluster of about 320 associated ORNs, and there are some 1500 aesthetascs per antennule (Grünert and Ache, 1988). The outer dendritic segment of the ORN is the site of olfactory transduction and consists of only plasma membrane surrounding microtubules and cytoplasm (no endoplasmic reticulum or mitochondria are present) (Grünert and Ache, 1988).

Both cAMP and IP_3 underlie lobster olfactory transduction

Individual odor molecules can either excite or inhibit a single ORN, but show both capabilities across the ORN population (McClintock and Ache, 1989; Michel *et al.*, 1991). These opposing effects are due to the activation of different conductances: a K^+ conductance mediates the suppressive effect (Michel *et al.*, 1991), while a non-selective cation conductance underlies excitation (Schmiedel-Jakob *et al.*, 1989; 1990).

These opposing conductances are mediated by cAMP and IP_3 (Figure 3) (review Fadool and Ache, 1994). Rapid kinetic measurements of second messenger production *in vitro* show the elevation, in the dendrites, of both cAMP and IP_3 in response to odor exposure (Boekhoff *et al.*, 1994) (Figure 4). Drugs targeting the cAMP pathway perturb the inhibitory response (Michel and Ache, 1992), and cAMP directly gates a K^+ conductance in ORN dendritic plasma membrane (Hatt and Ache, 1994), linking cAMP to the inhibitory response. The excitatory response is affected by drugs targeting the IP_3 pathway (i.e., heparin) (Fadool and Ache, 1992). Intracellular injection of IP_3 mimics the excitatory response (Fadool and Ache, 1992). IP_3 directly gates two conductances of different magnitudes in inside-out patches of cultured ORN somatic plasma membrane (Fadool and Ache, 1992) and ORN dendritic plasma membrane blebs (Hatt and Ache,

Figure 3. The lobster ORN dendritic plasma membrane supports at least two transduction pathways, one mediated by cAMP and one by IP_3. In both of these cases, odors activate the enzymes adenylyl cyclase or phospholipase C via a receptor/G protein cascade. The evoked messenger molecules then directly gate ion channels in the membrane. Na^+ entering the cell (possibly via the IP_3-gated channel) activates an additional membrane channel, and may serve to amplify the odor-evoked receptor potential. In the IP_3 cascade, diacylglycerol (DAG) is produced, along with IP_3, with the hydrolysis of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2).

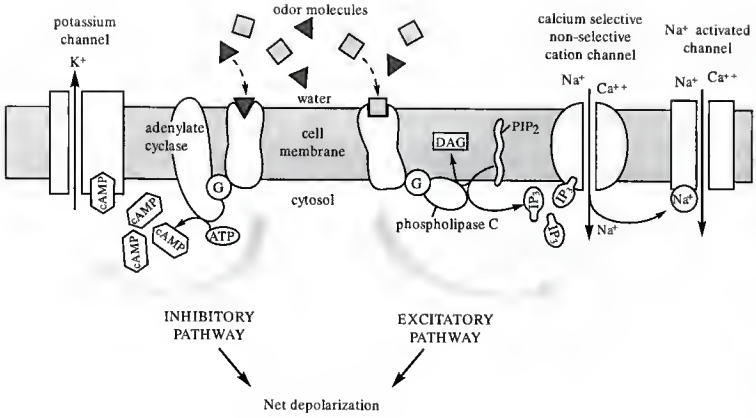
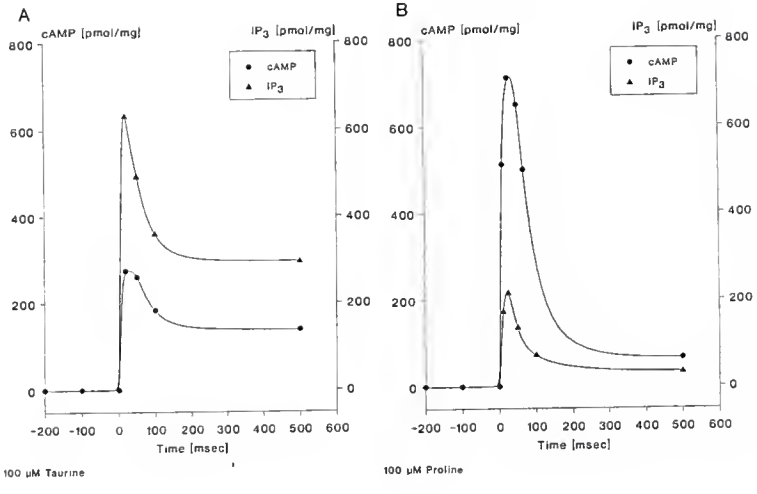

potassium channel
K+
odor molecules
water
adenylate cyclase
cell membrane
cytosol
G
ATP
cAMP
DAG
PIP2
phospholipase C
IP3
calcium selective non-selective cation channel
Na+
Ca++
Na+ activated channel
INHIBITORY PATHWAY
EXCITATORY PATHWAY
Net depolarization

Figure 4. Fast kinetic measurements of the second messengers cAMP and $1P_3$ in ORN dendrites as elicited by the odors taurine **(A)** and proline **(B)**. Dendritic membrane preparations were stimulated by individual odors before quenching the reactions and measuring second messenger levels by radioimmunoassay. Both odors elevate both messengers. However, the odor more likely to excite ORNs across the population (taurine) elevates $1P_3$ to a greater extent than cAMP, while the odor more likely to inhibit ORNs across the population (proline) elevates cAMP to a greater extent than IP_3. From Boekhoff *et al.*(1994), with permission.


A
cAMP [pmol/mg]
IP3 [pmol/mg]
cAMP
IP3
800
600
400
200
0
-200 -100 0 100 200 300 400 500 600
Time [msec]
100 µM Taurine
B
cAMP [pmol/mg]
IP3 [pmol/mg]
cAMP
IP3
800
600
400
200
0
-200 -100 0 100 200 300 400 500 600
Time [msec]
100 µM Proline

1994). When a cell-free patch containing a large conductance IP_3R was inserted into the cytoplasm of a second cultured ORN ("patch-cram"), application of odor to the ORN induced channel activity in the inside-out patch (Fadool and Ache, 1992), implying odor induced generation of IP_3 in the recipient ORN, an observation confirmed by Boekhoff and colleagues (1994). Together, these data implicate IP_3 as the "excitatory" second messenger.

The lobster olfactory IP_3 receptors

The IP_3-gated conductances described by Fadool and Ache (1990) and Hatt and Ache (1994) must be definitively localized to either the dendritic plasma membrane or to the endoplasmic membrane (the best understood location of IP_3Rs). This is an important point, as it would have great implications for the nature of the excitatory odor response: the elicited IP_3 could directly gate a plasma membrane channel, depolarizing the ORN by increasing the conductance of ions across the membrane, or it could release Ca^{2+} from intracellular stores, and this tertiary messenger would indirectly depolarize the ORN. While one could argue that patches from cultured ORN somata might include ER membrane, the patch recordings from dendritic blebs takes advantage of the morphological feature that the outer dendrite contains no membranous structures other than plasma membrane (Grünert and Ache, 1988). Additionally, the drug thapsigargin, which blocks the Ca^{2+}-ATPase responsible for refilling IP_3-depleted Ca^{2+} stores, did not affect the odor response (Fadool and Ache, unpublished results), as would be expected if this response were mediated by the release of Ca^{2+} from intracellular stores. It is therefore unlikely that the lobster olfactory IP_3Rs are localized to the endoplasmic reticulum.

Lobster ORNs express two different IP_3Rs. In inside-out patches from cultured ORN plasma membrane, the two IP_3-gated channels showed conductances of 74 pS (large

channel) and 30 pS (small channel) (Fadool and Ache, 1992); two IP_3-gated channels of similar conductances (64 pS and 27 pS) were recorded *in situ* from dendritic membrane (Hatt and Ache, 1994). The large and small conductance channels were found in the same patch of membrane only twice, and exhibited different voltage dependencies of the open probability (Fadool and Ache, 1992; Hatt and Ache, 1994). Both of these findings are consistent with the hypothesis that the two IP_3Rs are differentially expressed across the population of ORNs. Whether these two IP_3Rs are the products of different genes, splice variants of the same gene, or are modified (e.g. phosphorylated) forms of the same protein is unclear.

Structural similarity between the IP_3Rs in the plasma membrane of the lobster ORNs and the mammalian cerebellum is implied by immuno-crossreactivity. A polyclonal antibody, raised against the carboxyl-tail of the rat cerebellar type 1 IP_3R, increased the odor-evoked current over 400% when injected into the soma of cultured ORNs and, when copresented with 10^{-7} M IP_3, the antibody increased the probability of opening and the mean open time of both large and small conductance channels without affecting the channel conductance (Fadool and Ache, 1992).

Lobster olfactory IP_3Rs display important functional differences and similarities with vertebrate IP_3Rs. The lobster IP_3Rs are functionally insensitive to ATP (Fadool and Ache, 1992), even at high concentrations (up to 50 mM), unlike mammalian and amphibian IP_3Rs (Bezprozvanny and Ehrlich, 1994; Stehno-Bittel *et al.*, 1995). Preliminary evidence indicates that the lobster IP_3Rs mediate nonspecific cation conductances (Fadool and Ache, unpublished data) similar to intracellular IP_3Rs (Bezprozvanny and Ehrlich, 1994; Stehno-Bittel *et al.*, 1995).

G proteins mediate lobster olfactory transduction

As described above, both the cAMP and the IP_3 second messenger cascades rely on a heterotrimeric GTP-binding protein (G protein) composed of α-, β- and γ-subunits to transduce the signal between the ligand-activated receptor and a downstream metabolizing enzyme (e.g., adenylyl cyclase for cAMP or phospholipase C for IP_3). In lobster ORNs, the use of nonhydrolysable GTP analogs has demonstrated the necessity of such proteins in olfactory transduction (Fadool *et al.*, 1995). The identity of the G protein involved in the inhibitory odor response has not been identified [though it is clearly not the same as the one involved in the excitatory odor response (Fadool *et al.*, 1995)]. Two candidate α-subunits have been implicated in the excitatory odor response (Fadool *et al.*, 1995): a $G\alpha_{q/11}$ of the Gq family, and a $G\alpha_o$ of the Gi family. Only the $G\alpha_{q/11}$ protein has been localized to the ORN dendrites. These studies involved the use of antibodies to conserved regions of these proteins, so the G proteins that may function in the excitatory pathway must be isolated and characterized.

Specific Aims

In this project, I have attempted to address the following question: Is the excitatory odor response in lobster ORNs mediated by common components of an IP_3 signaling cascade? This dissertation presents a series of experiments designed to isolate and partially characterize two components of the lobster excitatory odor transduction pathway: an IP_3 receptor and a Gq protein. I have used a cloning strategy based on homology to known proteins to isolate and determine the primary structure of cDNAs for these two molecules from the lobster olfactory organ. I have demonstrated that these two molecules are expressed, as messenger RNAs, in the olfactory organ, a requirement for any protein involved in olfactory transduction. Together, these data demonstrate two fundamental principles: first, two transduction molecules, physiologically implicated in

the excitatory odor response, are present in the olfactory organ; and second, these molecules appear to share many features of primary structure with similar proteins in other IP_3 signaling cascades. These two molecules are the first components of the IP_3-mediated olfactory transduction pathway to have both structure and function characterized .

CHAPTER 2
MOLECULAR AND IMMUNOLOGICAL CHARACTERIZATION OF AN OLFACTORY ORGAN IP_3 RECEPTOR

Introduction

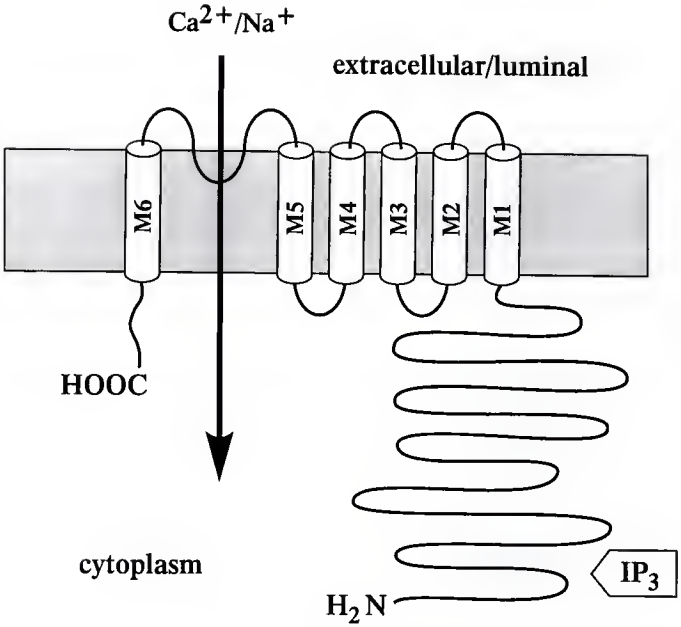
Physiological and biochemical evidence implicates inositol 1,4,5-trisphosphate (IP_3) as well as adenosine 3',5'-cyclic monophosphate (cAMP) as olfactory second messengers (reviews: Breer and Boekhoff, 1992; Ache, 1994). Though IP_3 plays a primary transduction role in many invertebrates, the precise functional role of IP_3 is less clear in vertebrate ORNs (e.g. Firestein *et al.*, 1991; Nakamura *et al.*, 1994; Kleene *et al.*, 1994; Nakamura *et al.*, 1996; Brunet *et al.*, 1996), where this messenger may serve as a modulatory factor, a required cofactor, or as an excitatory or inhibitory second messenger acting in parallel to the cAMP pathway. But in contrast to the cAMP transduction pathway, where many of the major molecular components have been cloned, sequenced and localized to the transduction zone (Jones and Reed, 1989; Bakalyar and Reed, 1990; Dhallan *et al.*, 1990; Bradley *et al.*, 1994; Liman and Buck, 1994), the IP_3 pathway has been minimally characterized at the molecular level. Notable exceptions include a phospholipase C from catfish (Abogadie *et al.*, 1995b) and Gq proteins from two species of lobster (Caribbean spiny lobster: Munger *et al.*, 1997; American lobster: Xu *et al.*, 1997) and *Drosophila* (Talluri *et al.*, 1995).

In olfactory systems, the binding of some odor molecules to G protein-associated receptors is thought to activate the enzyme phospholipase C, which hydrolyses the membrane lipid phosphatidylinositol 4,5-bisphosphate into IP_3 and diacylglycerol (Breer *et al.*, 1990; Boekhoff *et al.*, 1990; Berridge, 1993; Ronnett *et al.*, 1993; Boekhoff *et al.*, 1994). The IP_3 can then gate the intrinsic channel of IP_3 receptors (IP_3Rs) on the plasma

membrane of ORN cilia or outer dendrites (Fadool and Ache, 1992; Hatt and Ache, 1994; Restrepo *et al.*, 1990; Restrepo *et al.*, 1992; Lischka *et al.*, 1995). IP_3Rs have been traditionally thought to be principally localized to the ER and nuclear membranes (reviews: Furuichi *et al.*, 1994; Ferris and Snyder, 1992), but there is growing evidence for the presence of IP_3Rs in the plasma membrane (e.g. Restrepo *et al.*, 1990; Fadool and Ache, 1992; Fujimoto *et al.*, 1992; Restrepo *et al.*, 1992; Khan *et al.*, 1992; Sharp *et al.*, 1992; Hatt and Ache, 1994; Lischka *et al.*, 1994; Kuno *et al.*, 1994; Khan *et al.*, 1996) including the immunological localization of an IP_3R to the cilia (as well as other compartments) of rat ORNs (Cunningham *et al.*, 1993) and the description of a protein in catfish ORNs affinity labeled by an ^{125}I-IP_3 analogue (Kalinowski *et al.*, 1992); this latter protein, however, is half the size (107 kDa) of all other known IP_3Rs.

The primary structure of numerous vertebrate IP_3Rs has been determined by cDNA cloning (e.g. Yamada *et al.*, 1994; Yamamoto-Hino *et al.*, 1994; Maranto, 1994; Furuichi *et al.*, 1989; Mignery *et al.*, 1990; Sudhof *et al.*, 1991; Blondel *et al.*, 1993; Kume *et al.*, 1993) as well as *Drosophila* (Yoshikawa *et al.*, 1992). All of these cloned IP_3Rs are homologous, sharing the same basic structural motifs (Figure 5). Multiple isoforms, types 1, 2 and 3, have been identified in mammals; they possess 60-70% sequence identity and appear to vary only in sensitivity to modulation, affinity for IP_3 binding and tissue distribution. The IP_3R proteins consist of 2693-2833 amino acids, coded for by an mRNA of 9-10 kb, and have three functional domains: an IP_3-binding domain, a central modulatory domain, and an ion channel domain. The IP_3-binding domain lies within the first 650 amino acids of the IP_3R [amino acids 476-501 are labeled by photoaffinity ligands (Mourey *et al.*, 1993)]. The type 1 IP_3R contains the first of two short, alternatively spliced regions (S1) within the IP_3-binding domain. The modulatory domain lies between the IP_3-binding and channel domains, and may contain consensus sites for ATP binding and protein kinase A phosphorylation, along with several calcium related signals and the second

Figure 5. IP_3Rs all contain three principle domains: a ligand binding domain (IP_3) at the amino end of the protein, a central modulatory domain, and a channel domain with six transmembrane regions (M1-M6) and a putative pore-forming region. Both the amino and carboxyl termini are extend into the cytoplasm (this is true for receptors on the endoplasmic reticulum as well as on the plasma membrane). The channel is thought to be a nonselective cation channel. Modified from Mikoshiba, 1993.



After K. Mikoshiba, 1993.

splice site (SII; in type 1); the number and types of these consensus sites varies among IP_3R types and among species, and some sites (i.e., protein kinase C phosphorylation sites) are as yet undefined (review: Furuichi *et al.*, 1994). The channel domain contains six putative transmembrane regions [as defined by hydrophobicity and N-linked glycosylation sites (Michikawa *et al.*, 1994)] and a carboxyl tail thought to be involved in calcium release (Nakade *et al.*, 1991). The channel pore is thought to lie between the last two transmembrane regions, 5 and 6 (Michikawa *et al.*, 1994). It is the final two transmembrane regions and the carboxyl tail that show the greatest similarity in protein sequence to another intracellular calcium release channel, the ryanodine receptor. Although these cation channels are relatively nonselective (Bezprozvanny and Ehrlich, 1994; Stehno-Bittel *et al.*, 1995; but see McDonald *et al.*, 1993), their main role is thought to be the release of calcium from intracellular stores in response to an IP_3-mediated extracellular signal (review: Berridge, 1993).

Lobster olfactory IP_3Rs display important functional differences and similarities with vertebrate IP_3Rs. The lobster IP_3Rs are insensitive to ATP (Fadool and Ache, 1992), even at high concentrations (up to 50 mM), unlike mammalian and amphibian IP_3Rs (Bezprozvanny and Erhlich, 1993). Preliminary evidence indicates that the lobster IP_3Rs mediate nonspecific cation conductances, similar to intracellular IP_3Rs (Fadool and Ache, unpublished data).

Cunningham and colleagues (1993) provided evidence that rat ORNs express an IP_3R in cilia that is somewhat similar to the cerebellar type-1 IP_3R. But the antibodies used in these studies cross-react with all three IP_3R subtypes; so we cannot be sure whether the IP_3R in ORN cilia is the same as that expressed in cerebellum, or if the olfactory IP_3R is similar yet not identical IP_3R form. In this chapter, I describe the isolation of an IP_3R cDNA from the olfactory organ of the spiny lobster, *Panulirus argus*. This IP_3R is expressed in both olfactory organ and brain. Attempts to localize this IP_3R to the ORN dendrites--the site of olfactory transduction--are inconclusive, so the involvement of this

particular IP_3R is involved in shaping the initial odor response remains unproven. Portions of this work have previously appeared in abstract form (Munger *et al.*, 1995; Munger *et al.*, 1997).

Methods

RNA extraction: Olfactory organs (100 antennules per isolation) were harvested into a dry ice/ethanol bath, transported from the Florida Keys to the Whitney Lab on dry ice, and stored at -80°C until used. The antennules were homogenized in liquid nitrogen by mortar and pestle, then the powder was homogenized by polytron in a GTC/β-mercaptoethanol solution (sarkosyl added to 2% after homogenization). After removing cuticle by centrifugation, the homogenate was layered onto a 5.7M CsCl cushion for ultracentrifugation (24 hours, 20°C, 30,000 rpm in a Beckman SW-41 rotor). The RNA pellet was resuspended in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA-SDS, spectrophotometrically quantified, and stored in ethanol with 0.3 M sodium acetate, pH 5.5, at -20°C.

RT-PCR: IP_3R: Degenerate oligonucleotide primers were designed to match regions of conserved nucleotide sequence within the final putative transmembrane region (amino acids FFVMVIIIV, sense primer) and the carboxyl tail (amino acids EHNMWHY, antisense primer) of known IP_3Rs. Total RNA from nose (10 μg) was reverse transcribed with SuperScript II M-MLV reverse transcriptase, using the antisense primer. The resulting cDNA served as template for touchdown polymerase chain reaction (PCR) amplification. The PCR reaction mixture contained 100 pmol of each primer, 5% of the reverse transcription reaction volume, 2.5 units of a 60:1 mixture of *Taq:pfu* polymerases, 1.5 mM $MgCl_2$, 500 mM KCl, 100 mM Tris-HCl, pH 9.0 and 1% Triton X-100. The PCR cycling profile was as follows: [94°C, 5 min; 60°C 2 min; 72°C, 3 min] x 1 cycle; [94°C, 1 min; 59°C (- 1°C/cycle), 1 min; 72°C, 2 min] x 9 cycles; [94°C, 1

min; 52°C, 1 min; 72°C, 2 min] x 30 cycles. The resulting 203 bp product was gel purified, ligated into the TA-cloning vector pGem-T (Promega), and transformed into JM109 competent *E. coli* for subcloning. Control RNA samples, which were treated as above except for receiving no reverse transcriptase, showed no product. After screening colonies for appropriately sized inserts by PCR, individual clones were selected for sequencing by standard chain termination methods (Sanger *et al.*, 1977). Seven clones (78/79-1 through 78/79-7) were fully sequenced, and the cDNA sequences were translated, assembled and analyzed with GeneRunner (Hastings Software, Inc.) and BioImage DNA Sequence Film Reader software (BioImage). The deduced amino acid sequences were compared with all known proteins using BLAST software (NCBI, Altshul *et al.*, 1990).

3'-RACE (Rapid Amplification of cDNA Ends): The 3'-RACE technique is modified from Frohman *et al.* (1988). Total RNA from nose (10 μg) was reverse transcribed with an oligo-dT_{17} /Not I oligonucleotide. The resulting cDNA served as template for the first round of PCR amplification; the oligo-dT primer and a primer specific to clone 78/79-1 were used. The PCR product then served as template for a second round of PCR amplification in which the same oligo-dT primer and a nested 78/79-1-specific primer were used. The resulting product of 643 bp (clone 3R2A) was purified, subcloned and sequenced as described above.

Creation of cDNA minilibrary and library screening: A unidirectional cDNA minilibrary was constructed in the bacteriophage λgt22A vector system (Gibco-BRL Lambda System) from olfactory organ poly(A)+ RNA. cDNA ligated into the λ arms was reverse transcribed with the antisense degenerate primer (see above) coupled to a *Not I* adapter. The library was screened by plaque hybridization at high stringency (60°C) using clone 78/79-1 as probe. A 1707 bp clone (1111-2), which overlaps with clone 3R2A, was isolated. Clone 1111-2 maintains homology to other IP_3Rs. Three

more overlapping clones (56/25H3, 1272 bp, 62/25N2, 938 bp, and 3/144H2, 2386 bp) were also isolated from the library by PCR.

5'-RACE (Rapid Amplification of cDNA Ends): 5'-RACE was modified from Frohman *et al.* (1988) with the 5'-RACE kit (Life Technologies). Total RNA from nose (10 μg) was reverse transcribed as above with an IP_3R-specific antisense primer, and a poly(C)+ tail was added to the 3'-end of the antisense single strand cDNA with terminal transferase. The resulting cDNA served as template for the first round of PCR amplification using a nested antisense IP_3R-specific primer and an anchor primer to the poly(C)+ tail (*Sal I-Spe I*-GTACGGGIIGGGIIGGGIIG, where I = inosine). The same anchor primer and a second nested IP_3R-specific primer were then used in a second round of PCR amplification with the PCR products serving as template. The resulting products were purified, subcloned and sequenced as described above. Control RNA samples, which were treated as above, except that they received no reverse transcriptase or no terminal transferase, showed no product. PCR reagents for both reactions were: 50 pmol of each primer, 20% of the tailed cDNA (or 0.1% of the first PCR reaction), 1.5 mM $MgCl_2$, 500 mM KCl, 100 mM Tris-HCl, pH 9.0 and 1% Triton X-100. PCR cycling times were as follows: [94°C, 5 min; 49°C, 2 min; 72°C, 3 min] x 1 cycle; [94°C, 30 sec; 49°C, 30 sec; 72°C, 2 min] x 30 cycles. Sequencing, translation, assembly and analysis was done as above. A second 5'-RACE reaction was performed for the IP_3R to reach the 5'-UTR. Sequencing, translation, assembly and analysis was done as above; some clones were sequenced by the University of Florida's Interdisciplinary Center for Biotechnology DNA Sequencing Core Laboratory using the Taq DyeDeoxy Terminator and Dyeprimer Cycle sequencing protocols, developed by Applied Biosystems, and the Applied Biosystems Model 373A DNA Sequencer.

To control for errors which may have resulted from the actions of the DNA polymerases or the reverse transcriptase, four overlapping clones, which together spanned

the entire contig from the 5'- to the 3'-untranslated regions, were amplified in duplicate, independent RT-PCR reactions, and a consensus sequence was taken.

Ribonuclease Protection Assay (RPA): This assay was done according to the protocols accompanying the RPA II kit (Ambion). RNA samples (1.5 and 3 μg nose poly(A)+ RNA and 5 and 10 μg brain total RNA, along with yeast RNA controls) were hybridized in solution to a ^{32}P-labeled antisense riboprobe transcribed from clone 79/79-1. The RNA samples were then subjected to RNase digestion (RNase A and T1) and separated on an 8M urea/5% acrylamide gel. One of the yeast controls did not undergo RNase digestion. The gel was exposed to X-ray film for 18 days at -80°C with intensifying screens.

Results

cDNA cloning

The 203 bp cDNA product obtained from the initial RT-PCR was subcloned, sequenced, and conceptually translated. Seven separate clones (79/78-1 through 79/78-7) were sequenced and were found to be identical in nucleotide sequence. A comparison of the deduced amino acid sequence with sequences in the GenBank database revealed that these products are homologous to previously cloned IP_3Rs (they are less similar to the ryanodine receptors, which share some primary structure with IP_3Rs). The clone was extended to the 3'-untranslated region (UTR) by 3'-RACE (clone 3R2A). To obtain the remainder of the clone extending into the 5'-UTR, a cDNA minilibrary was constructed in λgt22A from olfactory organ poly(A)+ RNA reverse transcribed with the antisense degenerate primer used in the initial RT-PCR experiment. This library was screened by a combination of plaque hybridization and PCR to isolate a series of overlapping clones. The minilibrary apparently contained no clones that extended to the 5' UTR, so 5'-

RACE was used to isolate the remainder of the coding sequence. Four overlapping clones, which together spanned the entire open reading frame, were amplified in duplicate RT-PCR reactions (data not shown), and the consensus sequence across all three clones was taken. One of the original partial clones (1111-2) contained an intron that was not present in any of the subsequent confirmatory clones; this was the only intron sequence isolated from any of the clones, and no alternatively-spliced variant was ever isolated.

The assembled full-length clone has an open reading frame of 8409 bp coding for 2803 amino acids (Figure 6). The predicted protein has a calculated molecular weight of 320 kDa. The initiating methionine selected is the first methionine in the open reading frame, and a consensus intiation sequence (Kozak, 1991) is present at this point. The protein contains several residues predicted to be subject to posttranslational modification, including one consensus site for extracellular N-glycosylation (N^{2310}), three possible sites for phosphorylation by protein kinase A (S^{1001}, S^{1051}, S^{2509}, the latter of which is present in the predicted pore-forming region of the channel) and one putative site for tyrosine phosphorylation (Y^{1832}). All these sites await experimental evidence as to their relevance for the native protein. However, unlike many vertebrate IP_3Rs, there is no consensus sequence for ATP binding, though there is a related NAD/FAD consensus binding sequence.

The deduced amino acid sequence for the full-length clone is homologous to other IP_3Rs [Figure 7--NCBI sequence identification numbers in parentheses; *Drosophila*: 51% (266387); human type 1: 49% (1362832)]; it shows equivalent similarity to type 2 IP_3Rs, but is less similar to type 3 IP_3Rs, and is clearly only distantly related to ryanodine receptors, with the greatest similarity seen in the channel regions. Like other IP_3Rs, the putative pore-forming region of the channel domain (between the fifth and sixth membrane-spanning regions), is poorly conserved. The entire lobster IP_3R sequence seems to maintain the distribution of conserved and variable regions that has been seen in the other IP_3Rs. One portion of the sequence, however, stands out as being

Figure 6. The nucleotide and deduced amino acid sequence of the lobster IP_3R cDNA. The coding region of 8409 bp encodes 2803 amino acids. The target sites of the degenerate primers used in the initial isolation are indicated in gray with arrows. The six predicted transmembrane regions (M1-M6) are indicated by underlines. Consensus motifs for one putative N-glycosylation site (arrowhead), 3 potential sites for protein kinase A phosphorylation (stars) and a putative tyrosine phosphorylation site (filled circle) are seen.

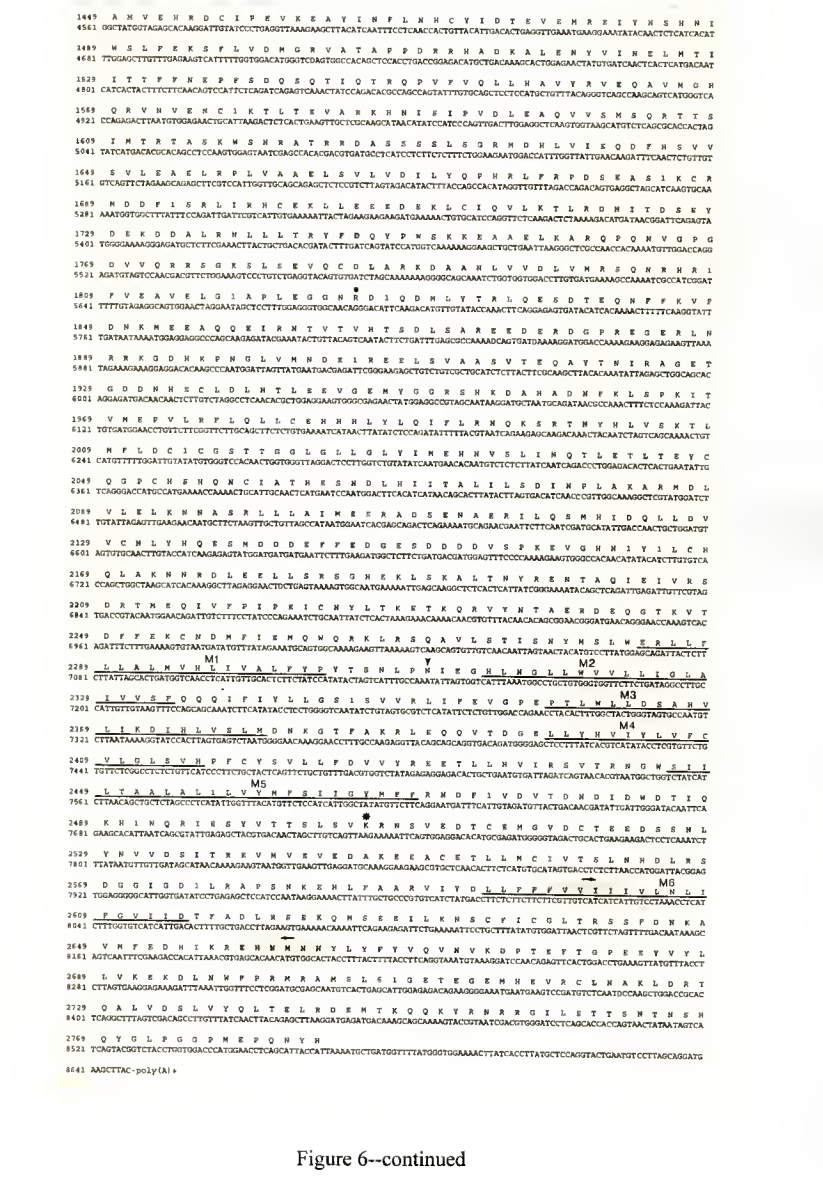
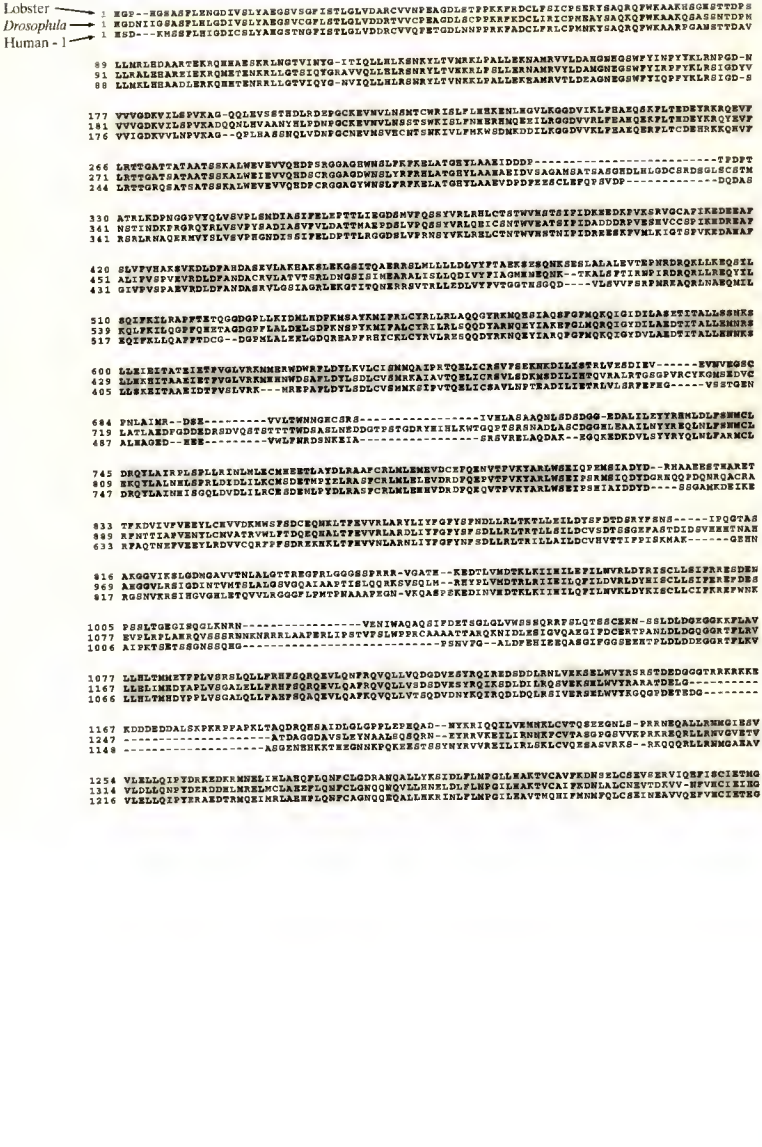


Figure 6--continued

Figure 7. The lobster IP_3R protein is aligned with an IP_3R from *Drosophila* (NCBI ID: 266387) and a type 1 IP_3R from human (NCBI ID: 1362832). Shading indicates amino acids conserved between the lobster protein and at least one of the other two proteins. The six transmembrane regions (M1-M6) are indicated by underlining. A unique hydrophilic region, rich in lysine residues, is seen at amino acids 1159-1186.


Lobster
Drosophila
Human - 1

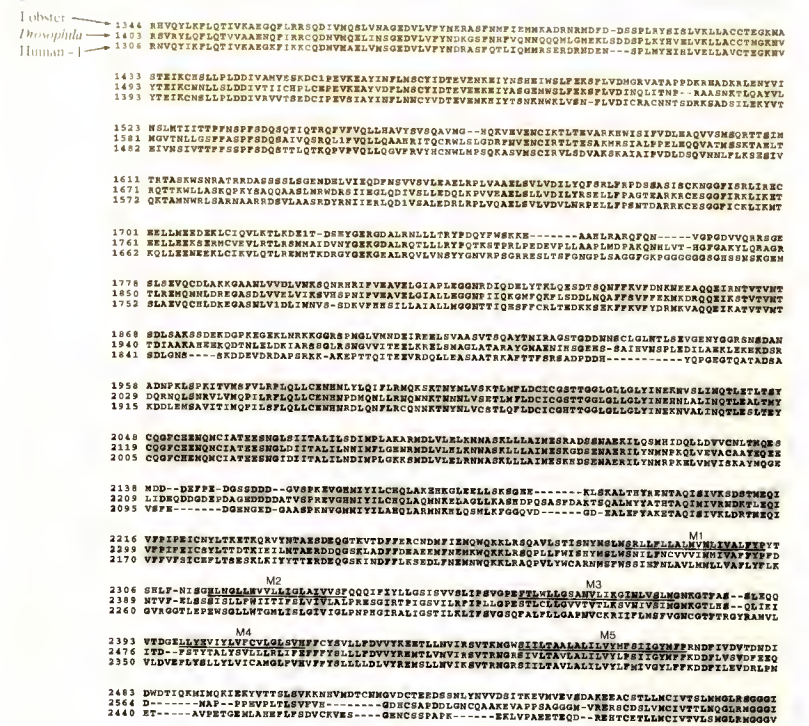


Figure 7--continued

completely unrepresented in the other IP_3Rs identified to date: amino acids 1159-1186, a lysine-rich, hydrophilic stretch, is not present in any other IP_3R (Figure 7). Searches of GenBank with this amino acid sequence revealed no matches with known proteins which might unveil a possible function for this portion of the protein.

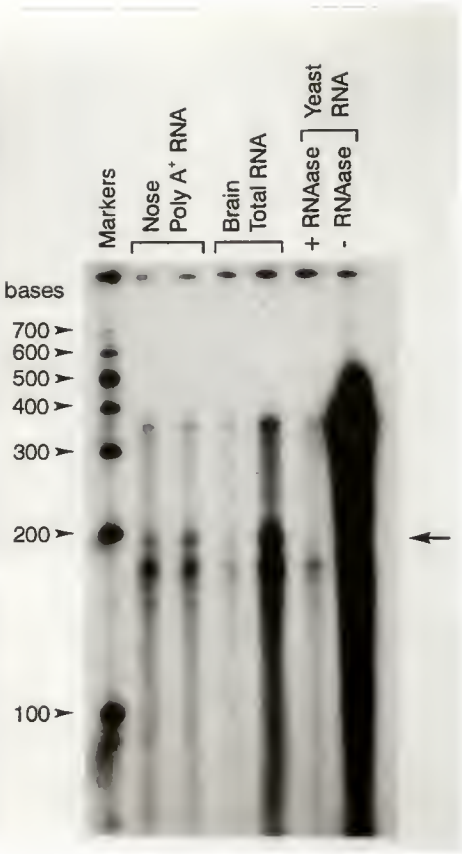
Ribonuclease Protection Assay

To verify that the source of the IP_3R cDNA was indeed olfactory organ RNA, a ^{32}P-labeled riboprobe representing a portion of the cloned IP_3R ribonuclease protection assay was used to probe for the IP_3R message in extracts of olfactory organ poly(A)+ RNA. A riboprobe translated from the original 203 bp product was incubated with olfactory organ total RNA under high stringency conditions, producing a protected band of the appropriate size (Figure 8). A protected band was also seen in an assay of total RNA from brain. No protected bands of the appropriate size were seen when control hybridizations were done with yeast RNA controls.

Discussion

The cDNA identified in this study shows much structural similarity to known vertebrate and invertebrate IP_3Rs. As with other IP_3Rs, the greatest conservation is seen at the amino end, the location of the IP_3-binding domain, and the carboxyl end, the site of the channel portion of the molecule. A greater degree of variability is seen in the central, modulatory domain. Six hydrophobic domains correspond to the putative transmembrane regions of other IP_3Rs (Figure 7). The putative pore-forming region between the final two transmembrane regions, M5 and M6 (Michikawa *et al.*, 1994), shows almost no sequence conservation: why a family of channels with a presumably similar ion selectivity exhibit such divergence in the pore sequence is unknown. There are three protein kinase A substrate consensus motifs, numerous protein kinase C and casein kinase II motifs, as well as one extracellular site for N-glycosylation and a site for tyrosine

Figure 8. A ribonuclease protection assay, probed with a 203 bp riboprobe transcribed from a portion of the lobster IP_3R. An appropriate sized protected band is seen in nose poly(A)+ RNA (1.5 and 3 μg) and in brain total RNA (5 and 10 μg) (indicated by arrow), but not in the yeast + RNase control. A strong, smaller sized band is seen both in the experimental and the yeast control lanes. A yeast control with no RNase treatment shows the undigested probe; residual undigested probe is seen in all experimental lanes running at 350 bp.


Markers
Nose
Poly A+ RNA
Brain
Total RNA
+ RNAase
- RNAase
Yeast
RNA
bases
700
600
500
400
300
200
100

phosphorylation; whether or not these sites are functional in the native protein remains to be determined. The IP_3R encoded by this cDNA is structurally consistent with those characterized by Fadool and Ache (1992) and Hatt and Ache (1994). Both groups saw channels with similar physiological and pharmacological properties to those of ER IP_3Rs (e.g. Bezprozvanny and Erhlich, 1994), including similar conductances and a sensitivity to heparin. However, the lobster olfactory IP_3Rs are insensitive to ATP (Fadool and Ache, 1992), which is known to modulate vertebrate IP_3Rs by increasing the channel open probability (Bezprozvanny and Erhlich, 1993). The cloned lobster IP_3R described here has no consensus motif for ATP-binding, though it does share an NAD/FAD-binding site (Sternberg and Taylor, 1984; Zheng *et al.*, 1993) with other IP_3Rs (the *Drosophila* IP_3R also lacks an apparent ATP-binding site, but its ATP sensitivity is unknown). This insenstivity to ATP may be typical of calcium-release channels in lobsters, or arthropods in general, rather than be representative of plasma membrane IP_3Rs, as a ryanodine receptor characterized in American lobster is also insenstive to ATP (Seok *et al.*, 1992).

The ribonuclease protection assay shows that the IP_3R message is expressed in both olfactory organ and brain (Figure 9), though at much higher levels in the latter. As the probe was directed against a somewhat conserved region of the coding sequence, it is possible that these tissues are expressing similar, but different, messages. Proteins need not be olfactory-specific to be important for olfactory transduction: expression in neural tissue outside the nose is seen for several olfactory transduction-related genes, including G_{olf} (Drinnan *et al.*, 1991) and the cAMP-gated channel (Kingston *et al.*, 1996).

One region of the lobster cDNA does represent a protein sequence unique to IP_3Rs (Figure 7). Although the function of this peptide is unknown, it does resemble some nuclear localization signals (Dingwall and Laskey, 1991); IP_3Rs have been localized to nuclear membranes (e.g., Sullivan *et al.*, 1995) This region also contains a protein kinase C consensus phosphorylation site (K1160). Heterologous expression of this cDNA will not only allow the characterization of this IP_3R, but also the functional analysis of this unique

region. It is also critical to determine whether the spiny lobster expresses other IP_3Rs, and if so, whether these other IP_3Rs share this same unusual sequence.

Though this IP_3R shares some structural features consistent with the functional properties of those characterized by Fadool and Ache (1992) and Hatt and Ache (1994), it remains unproven that this cDNA encodes one of those proteins. There are no known structural motifs indicating that a particular protein is involved in olfactory transduction. The promoter region of the gene for this IP_3R is uncharacterized, so whether it contains an olfactory system-related transcription factor-binding site as is seen in vertebrates with Olf-1 (Wang and Reed, 1993; Wang *et al.*, 1993) is unknown. While integral membrane proteins contain signal sequences for insertion into membranes, none that differentiate between endoplasmic reticulum and the plasma membrane are known. However, there is good evidence that plasma membrane IP_3Rs are similar in structure (e.g., Cunningham *et al.*, 1993), if not identical (Khan *et al.*, 1996) to those localized to the ER. Therefore, the similarity of the lobster IP_3R to other IP_3Rs cannot be seen as evidence for localization to either the endoplasmic reticulum or plasma membrane. Antibodies against the encoded protein must now be used to localize it to cellular compartments within the ORNs; in addition, the channel must be heterologously expressed so that its functional properties can be compared with those of the native channels.

CHAPTER 3
MOLECULAR CHARACTERIZATION OF AN OLFACTORY ORGAN GQ PROTEIN

Introduction

The transduction of many extracellular stimuli, such as neurotransmitters or light, can be mediated by a signaling pathway that links receptors and downstream effectors (e.g., ion channels or enzymes). Heterotrimeric GTP-binding proteins (G proteins) often serve to couple these two signaling components (Neer, 1995; Hamm and Gilchrist, 1996). Composed of α, β and γ subunits, these G proteins bind to the activated receptor as well as affect the downstream enzyme or channel, with many of the targeted enzymes synthesizing second messengers (e.g. IP_3 or cyclic nucleotides). The α subunits are the best characterized of the subunits; they have been classified into four categories based on sequence homologies: Gs, Gi, Gq and G_{12} (each family contains numerous members; Neer, 1995). Although the α subunit was previously thought to be exclusively responsible for the signaling capabilities of the G protein trimer, it is now established that the $\beta\gamma$ complex can itself activate downstream effectors in many systems (Clapham and Neer, 1993; Hamm and Gilchrist, 1996).

No G protein has yet been functionally implicated in olfactory transduction. Well before the discovery of a family of putative olfactory receptors (Buck and Axel, 1991)--all members of a superfamily of G protein-coupled receptors that includes rhodopsin--the mediation of at least some aspects of olfactory transduction by G proteins was established (Pace *et al.*, 1985; Sklar *et al.*, 1986; Bruch and Kalinowski, 1987; Bruch, 1990). A vertebrate G protein α-subunit, G_{olf} (a member of the Gs family), has been cloned and localized to the cilia of the olfactory receptor neurons (ORNs; Jones and Reed, 1989).

G_{olf} is thought to activate a type III adenylyl cyclase that sythesizes the second messenger adenosine 3',5'-cyclic monophosphate (cAMP); cAMP can then activate a cyclic nucleotide-gated (CNG) channel in the ORN cilia plasma membrane. Other G protein α-subunits are present in the vertebrate olfactory epithelium (Jones *et al.*, 1988; Jones and Reed, 1989; Menco *et al.*, 1992; Abogadie *et al.*, 1995a; Dellacorte *et al.*, 1996); some of them are unlikely to couple to adenylyl cyclase. In both vertebrates and invertebrates IP_3 (in addition to cAMP) serves as an olfactory second messenger (Restrepo *et al.*, 1990; Breer *et al.*, 1990; Boekhoff *et al.*, 1990; Bruch and Teeter, 1990; Dionne, 1992; Ivanova and Caprio, 1992; Miyamoto *et al.*, 1992; Michel and Ache, 1992; Fadool and Ache, 1992; Lucero *et al.*, 1992; Ronnett *et al.*, 1993; Bacigalupo *et al.*, 1993; Lischka *et al.*, 1995; Boekhoff *et al.*, 1994; Lucero and Piper, 1994). Therefore, more than one G protein α-subunit subtype might be involved in olfactory transduction. In the spiny lobster ORNs, odor-evoked elevations of cAMP and IP_3 mediate opposing conductances, with cAMP triggering the inhibitory outward current, and IP_3 the excitatory inward current (Fadool and Ache, 1992; Michel and Ache, 1994; Boekhoff *et al.*, 1994). Recently, Fadool and colleagues (1995) used electrophysiological and biochemical techniques to demonstrate that different G proteins mediate these two conductances. Although the G protein involved in the inhibitory pathway remains unknown, two different α-subunits were implicated in excitatory odor transduction: a $G\alpha_o$ from the Gi family and a $G\alpha_q$ or $G\alpha_{11}$ from the Gq family.

The precise nature of this Gq-like α-subunit must be characterized. In this chapter, I present evidence for the presence of a Gq-like protein in the lobster olfactory organ; this protein is consistent in structure and localization with the Gq-like α-subunit previously implicated in the excitatory odor response in the spiny lobster, *Panulirus argus*.

Portions of this work have previously appeared in abstract form (Munger *et al.*, 1997).

Methods

RNA Extraction: Olfactory organs (100 antennules per isolation) were harvested into a dry ice/ethanol bath, transported from the Florida Keys to the Whitney Lab on dry ice, and stored at -80°C until used. The antennules were homogenized in liquid nitrogen by mortar and pestle, then the powder was homogenized by polytron in a GTC/β-mercaptoethanol solution (sarkosyl added to 2% after homogenization). After removing cuticle by centrifugation, the homogenate was layered onto a 5.7M CsCl cushion for ultracentrifugation (24 hours, 20°C, 30,000 rpm in a Beckman SW-41 rotor). The RNA pellet was resuspended in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA-SDS, spectrophotometrically quantified and stored in ethanol with 0.3 M sodium acetate, pH 5.5, at -20°C.

RT-PCR: RT-PCR experiments were done as described in Chapter 2 Methods except as noted. Briefly, degenerate oligonucleotide primers were designed to a portion of the Switch II region common to Gα's [amino acids KWIHCFE, sense primer AA(AG) TGG AT(ATC) CA(CT) TG(TC) TT(TC) GA] and to the common carboxyl tail of $G\alpha_q$ and $G\alpha_{11}$ [amino acids KEYNLV, antisense primer (ACTG)AC IA(AG) (AG)TT (AG)TA (TC)TC (TC)TT, where I=inosine]. Total RNA from nose (10μg) was reverse transcribed, as described, using the antisense primer. Each primer (100 pmol), along with 1/20 of the reverse transcription reaction, was used in a touchdown PCR. The PCR cycling profile was as follows: [94°C, 5 min; 50°C, 2min; 72°C, 1.5 min] x 1 cycle; [94°C, 30 sec; 49°C (-1°C/cycle), 30 sec; 72°C, 1.5 min] x 9 cycles; [94°C, 30 sec; 40°C, 30 sec; 72°C, 1.5 min] x 30 cycles. The PCR product was diluted 1:1000 and served as template for a second, identical PCR. The resulting 435 bp product was gel purified, ligated into the plasmid pGem-T, and transformed into JM109 cells for subcloning.

Control RNA samples, which were treated as above except for receiving no reverse transcriptase, showed no product. After colonies were screened by PCR for inserts of appropriate size, individual clones were selected for sequencing by standard chain termination methods. One clone (RTG-4) was fully sequenced.

3'-RACE (Rapid Amplification of cDNA Ends): The 3'-RACE technique was performed as described in Chapter 2 Methods, except as noted. After reverse transcribing 10 μg of olfactory organ total RNA with an oligo-dT_{17} oligonucleotide, the resulting cDNA served as template for the first round of PCR amplification; one primer was directed against a portion of the oligo-dT primer, the second was specific to clone RTG-4. The PCR product then served as template for a second round of PCR amplification using the a nested oligo-dT primer and a nested RTG-specific primer. The resulting product of 250 bp was purified, subcloned and sequenced as described above. Control RNA samples, which were treated as above except that no reverse transcriptase was added, showed no product.

5'-RACE (Rapid Amplification of cDNA Ends): 5'-RACE was performed as described, except as noted. Total RNA from nose (10 μg) was reverse transcribed as above using an RTG-4-specific antisense primer, and a poly(C)+ tail was added to the 3'-end of the antisense single strand cDNA with terminal transferase. The resulting cDNA served as template for the first round of PCR amplification; a nested antisense RTG-4-specific primer and an anchor primer to the poly(C)+ tail were used. These PCR products then served as template for a second round of PCR amplification where the same anchor primer and a second nested RTG-4-specific primer were. The resulting products were purified, subcloned and sequenced as described above. Control RNA samples, which were treated as above except for receiving no reverse transcriptase or no terminal transferase, showed no product. PCR cycling times were as follows: [94°C, 5 min; 49°C, 2 min; 72°C, 3 min] x 1 cycle; [94°C, 30 sec; 49°C, 30 sec; 72°C, 2 min] x 30

cycles. Sequencing, translation, assembly and analysis was done as above. A single clone, 5RH1, was fully sequenced.

To control for errors that may have resulted from the actions of the DNA polymerases or the reverse transcriptase, two independent clones, which spanned the entire contig from the 5'- to the 3'-untranslated regions, were amplified in duplicate RT-PCR reactions, sequenced, and a consensus sequence translated for analysis.

Northern Hybridization: Total RNA from nose (150 µg) was denatured with 15% glyoxal:DMSO (1:1) then run overnight at 20 V on a 1.1% agarose gel in sodium phosphate buffer (pH 7.0). The gel was blotted to MagnaPlus nylon (MSI) by capillary action in 20x SSC. The blotted RNA was UV-crosslinked to the membrane. The membrane was prehybridized for 1 hour at 60°C in: 50% formamide, 5x SSPE, 5x Denhardt's reagent, 0.5% SDS and 100 µg/ml herring sperm DNA. The membrane was then hybridized to a ^{32}P-labeled riboprobe (transcribed from clone RTG-4) overnight in fresh hybridization solution. The next day, the membrane was washed 3 x 15 min in 1x SSPE/0.5% SDS and 1X 15 min in 0.1x SSPE/0.5% SDS, both at 60°C. The membrane was exposed to X-ray film for 4 days at -80°C with intensifying screens.

Results

cDNA cloning

Using RT-PCR and degenerate oligonucleotide primers, I was able to amplify a 435 bp cDNA product; this product was subcloned, sequenced, and conceptually translated. A comparison of the deduced amino acid sequence with sequences in the GenBank database revealed that this product was homologous to members of the Gq family of G proteins. This product was extended to the 5'-UTR by 5'-RACE, and to the 3'-UTR by 3'-RACE. To control for possible sequence errors induced by the reverse

transcriptase or DNA polymerases, two additional independent full length clones were isolated by RT-PCR (data not shown), and the consensus sequence across all three clones was taken.

The assembled full-length clone has an open reading frame of 1059 bp coding for 353 amino acids (Figure 9). The predicted protein has a calculated molecular weight of 41.5 kDa. The deduced amino acid sequence for the full-length clone shows a high degree of identity to other known $G\alpha_q$ proteins [Figure 10--NCBI sequence identifications in parentheses; *Drosophila*: 84% (944923); *Limulus*: (Munger *et al.*, in press; mouse: 82% (193502)], and is less similar to other G types [e.g. bovine $G\alpha_{i1}$: 47% (121019)]. The position of the initiating methionine (Figure 9) was selected for several reasons: it is the first methionine in the open reading frame; there is a well conserved consensus initiation sequence (Kozak, 1991); initiating transcription at this position results in a protein of identical length to most Gq proteins (Figure 10).

Sequence analysis of the lobster Gq protein shows it to contain several motifs characteristic of other Gq proteins (Figure 9; Strathmann and Simon, 1990; Simon *et al*, 1991; Wedegaertner *et al.*, 1995), including putative sites for palmidylation, two N-terminal cysteines (CC4); an absence of N-terminal myristylation sites (though putative myristylation sites do exist at several sites in the middle of the protein); a putative cholera toxin ADP ribosylation site (R177), but no cysteine at the C-terminal subject to pertussis toxin ADP ribosylation; and the G40TGES "GAG-box" sequence which is present in the GTP-binding domain of other Gq proteins. The sequences of the Switch I, II and III regions, all involved in GTP-dependent conformational changes of the α-subunit (Lambright *et al.*, 1994), are all highly conserved.

Figure 9. The nucleotide and deduced amino acid sequence of the cloned lobster Gq cDNA. The 1059 bp coding region encodes a 353 amino acid protein. Gray shading and arrows indicate the sequences to which the degenerate PCR primers were designed. Also indicated are consensus motifs for cholera toxin-dependent ADP ribosylation (filled circle) and palmidylation (arrowheads), as well as the "GAG" box (box), all characteristic of Gq proteins. Amino acids in common with the $G\alpha_o$ decapeptide (LDRIGAADYQ) that served as antigen for one antibody that blocked the excitatory odor response (Fadool *et al.*, 1995) are indicated with stars.

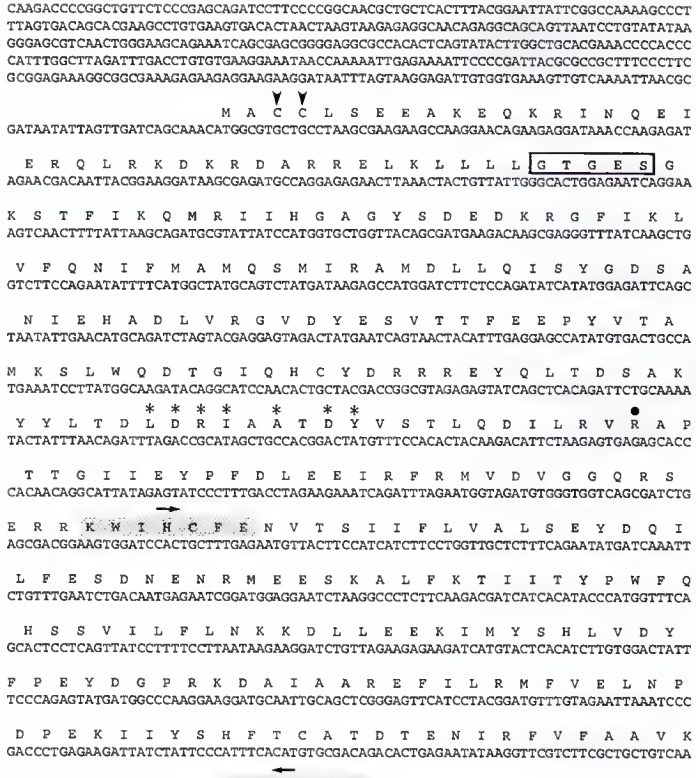
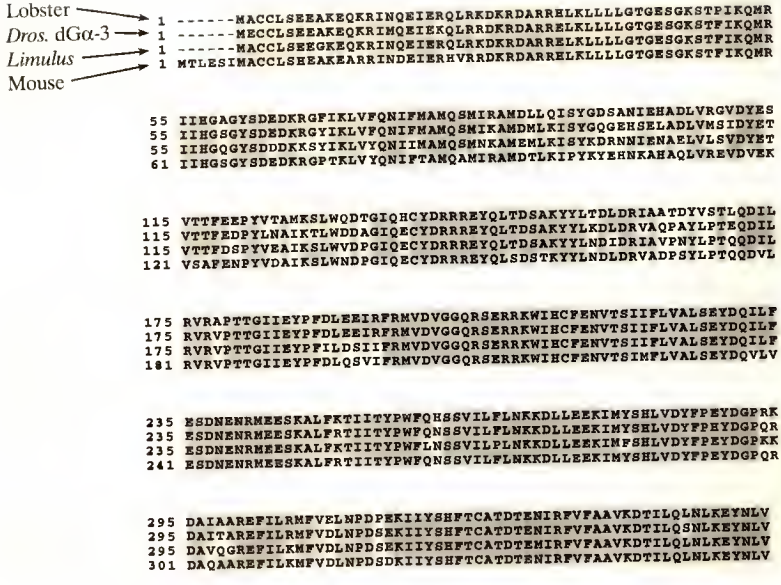

1 CAAGACCCCGGCTGTTCTCCCGAGCAGATCCTTCCCCGGCAACGCTGCTCACTTTACGGAATTATTCGGCCAAAAGCCCT
81 TTAGTGACAGCACGAAGCCTGTGAAGTGACACTAACTAAGTAAGAGAGGCAACAGAGGCAGCAGTTAATCCTGTATATAA
161 GGGAGCGTCAACTGGGAAGCAGAAATCAGCGAGCGGGGAGGCGCCACACTCAGTATACTTGGCTGCACGAAACCCCACCC
241 CATTTGGCTTAGATTTGACCTGTGTGAAGGAAATAACCAAAAATTGAGAAAATTCCCCGATTACGCGCCGCTTTCCCTTC
321 GCGGAGAAAGGCGGCGAAAGAGAAGAGGAAGAAGGATAATTTAGTAAGGAGATTGTGGTGAAAGTTGTCAAAATTAACGC
1 M A C C L S E E A K E Q K R I N Q E I
401 GATAATATTAGTTGATCAGCAAACATGGCGTGCTGCCTAAGCGAAGAAGCCAAGGAACAGAAGAGGATAAACCAAGAGAT
20 E R Q L R K D K R D A R R E L K L L L L G T G E S G
481 AGAACGACAATTACGGAAGGATAAGCGAGATGCCAGGAGAGAACTTAAACTACTGTTATTGGGCACTGGAGAATCAGGAA
46 K S T F I K Q M R I I H G A G Y S D E D K R G F I K L
561 AGTCAACTTTTATTAAGCAGATGCGTATTATCCATGGTGCTGGTTACAGCGATGAAGACAAGCGAGGGTTTATCAAGCTG
73 V F Q N I F M A M Q S M I R A M D L L Q I S Y G D S A
641 GTCTTCCAGAATATTTTCATGGCTATGCAGTCTATGATAAGAGCCATGGATCTTCTCCAGATATCATATGGAGATTCAGC
100 N I E H A D L V R G V D Y E S V T T F E E P Y V T A
721 TAATATTGAACATGCAGATCTAGTACGAGGAGTAGACTATGAATCAGTAACTACATTTGAGGAGCCATATGTGACTGCCA
126 M K S L W Q D T G I Q H C Y D R R R E Y Q L T D S A K
801 TGAAATCCTTATGGCAAGATACAGGCATCCAACACTGCTACGACCGGCGTAGAGAGTATCAGCTCACAGATTCTGCAAAA
153 Y Y L T D L D R I A A T D Y V S T L Q D I L R V R A P
881 TACTATTTAACAGATTTAGACCGCATAGCTGCCACGGACTATGTTCCACACTACAAGACATTCTAAGAGTGAGAGCACC
180 T T G I I E Y P F D L E E I R F R M V D V G G Q R S
961 CACAACAGGCATTATAGAGTATCCCTTTGACCTAGAAGAAATCAGATTTAGAATGGTAGATGTGGGTGGTCAGCGATCTG
206 E R R K W I H C F E N V T S I I F L V A L S E Y D Q I
1041 AGCGACGGAAGTGGATCCACTGCTTTGAGAATGTTACTTCCATCATCTTCCTGGTTGCTCTTTCAGAATATGATCAAATT
233 L F E S D N E N R M E E S K A L F K T I I T Y P W F Q
1121 CTGTTTGAATCTGACAATGAGAATCGGATGGAGGAATCTAAGGCCCTCTTCAAGACGATCATCACATACCCATGGTTTCA
260 H S S V I L F L N K K D L L E E K I M Y S H L V D Y
1201 GCACTCCTCAGTTATCCTTTTCCTTAATAAGAAGGATCTGTTAGAAGAGAAGATCATGTACTCACATCTTGTGGACTATT
286 F P E Y D G P R K D A I A A R E F I L R M F V E L N P
1281 TCCCAGAGTATGATGGCCCAAGGAAGGATGCAATTGCAGCTCGGGAGTTCATCCTACGGATGTTTGTAGAATTAAATCCC
313 D P E K I I Y S H F T C A T D T E N I R F V F A A V K
1361 GACCCTGAGAAGATTATCTATTCCCATTTCACATGTGCGACAGACACTGAGAATATAAGGTTCGTCTTCGCTGCTGTCAA
340 D T I L Q L N L K E Y N L V
1441 AGACACAATTCTGCAGCTAAATCTAAAGGAATACAACTTGGTGTAATGTAGAGTTATGCCCAACTGGCTGAGAGTGAACA
1521 TTAGATGAAGTGACAGCATGTATCCTTCTCAGTGTAGGGGCTGTGACCTAAGGACATTTCTTCATCACCCCTTACATGAC
1601 ACCATACCAATTTTGTAAACAAGGCAGTCATATTTTGCATCTACAAACCCATATTCAGTGTCCCAAAGAAATAAAGGTTC
1681 TATGTTGTT-poly(A)+

Figure 10. Alignment of the lobster Gq protein with Gq proteins (NCBI ID in parentheses) from *Drosophila* ($dG_q\alpha$-3: 944923), *Limulus* (Munger *et al.*, in press), and mouse (193502). Shading indicates amino acids conserved between the lobster protein and at least one of the other three proteins.


Lobster
Dros. dGα-3
Limulus
Mouse
1 ------MACCLSEEAKEQKRINQEIERQLRKDKRDARRELKLLLLGTGESGKSTFIKQMR
1 ------MECCLSEEAKEQKRIMQEIEKQLRRDKRDARRELKLLLLGTGESGKSTFIKQMR
1 ------MACCLSEEGKEQKRINQEIERQLRKDKRDARRELKLLLLGTGESGKSTFIKQMR
1 MTLESIMACCLSHEAKEARRINDEIERHVRRDKRDARRELKLLLLGTGESGKSTFIKQMR
55 IIHGAGYSDEDKRGFIKLVFQNIFMAMQSMIRAMDLLQISYGDSANIEHADLVRGVDYES
55 IIHGSGYSDEDKRGYIKLVFQNIFMAMQSMIKAMDMLKISYGQGEHSELADLVMSIDYET
55 IIHGQGYSDDDKKSYIKLVYQNIIMAMQSMNKAMEMLKISYKDRNNIENAELVLSVDYET
61 IIHGSGYSDEDKRGPTKLVYQNIFTAMQAMIRAMDTLKIPYKYEHNKAHAQLVREVDVEK
115 VTTFEEPYVTAMKSLWQDTGIQHCYDRRREYQLTDSAKYYLTDLDRIAATDYVSTLQDIL
115 VTTFEDPYLNAIKTLWDDAGIQECYDRRREYQLTDSAKYYLKDLDRVAQPAYLPTEQDIL
115 VTTFDSPYVEAIKSLWVDPGIQECYDRRREYQLTDSAKYYLNDIDRIAVPNYLPTQQDIL
121 VSAFENPYVDAIKSLWNDPGIQECYDRRREYQLSDSTKYYLNDLDRVADPSYLPTQQDVL
175 RVRAPTTGIIEYPFDLEEIRFRMVDVGGQRSERRKWIHCFENVTSIIFLVALSEYDQILF
175 RVRVPTTGIIEYPFDLEEIRFRMVDVGGQRSERRKWIHCFENVTSIIFLVALSEYDQILF
175 RVRVPTTGIIEYPFILDSIIFRMVDVGGQRSERRKWIHCFENVTSIIFLVALSEYDQILF
181 RVRVPTTGIIEYPFDLQSVIFRMVDVGGQRSERRKWIHCFENVTSIMFLVALSEYDQVLV
235 ESDNENRMEESKALFKTIITYPWFQHSSVILFLNKKDLLEEKIMYSHLVDYFPEYDGPRK
235 ESDNENRMEESKALFRTIITYPWFQNSSVILFLNKKDLLEEKIMYSHLVDYFPEYDGPQR
235 ESDNENRMEESKALFKTIITYPWFLNSSVILPLNKKDLLEEKIMFSHLVDYFPEYDGPKK
241 ESDNENRMEESKALFRTIITYPWFQNSSVILFLNKKDLLEEKIMYSHLVDYFPEYDGPQR
295 DAIAAREFILRMFVELNPDPEKIIYSHFTCATDTENIRFVFAAVKDTILQLNLKEYNLV
295 DAITAREFILRMFVDLNPDSEKIIYSHFTCATDTENIRFVFAAVKDTILQSNLKEYNLV
295 DAVQGREFILKMFVDLNPDSEKIIYSHFTCATDTEMIRFVFAAVKDTILQLNLKEYNLV
301 DAQAAREFILKMFVDLNPDSDKIIYSHFTCATDTENIRFVFAAVKDTILQLNLKEYNLV

Northern Hybridization

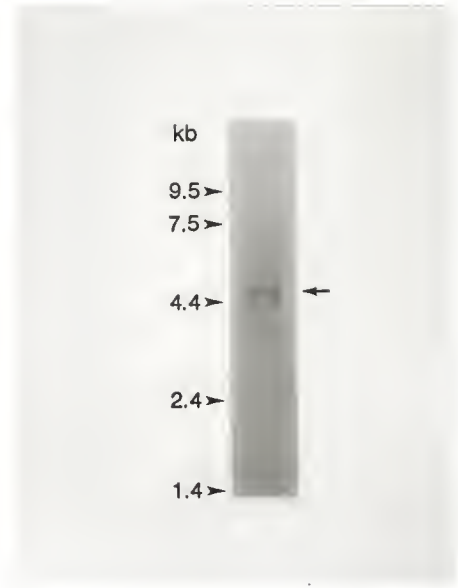
A Northern blot of lateral antennule total RNA was probed with a riboprobe transcribed from clone RTG-4 (nucleotides 1049-1483 of the complete clone) at high stringency. A single band of 4.6 kb is seen (Figure 11).

Discussion

The cDNA identified in this study shows the greatest similarity to known vertebrate and invertebrate Gq proteins, particularly the $G\alpha_q$ and $G\alpha_{11}$ types (Figure 9). The Gq family of α-subunits is highly conserved in primary structure. This high degree of identity allows little speculation about the functional consequences of structural motifs. While a few basic regions mediating some functions of the G protein α-subunit have been identified (Neer, 1994), such as interaction with the βγ complex at the amino-end of the α-subunit, or interaction with the receptor at the carboxyl end, these regions are not well defined, and it is difficult to pair a particular effector or receptor to an α-subunit, simply by examining primary structure. Therefore, the isolated cDNA might, or might not, encode an "olfactory" or "sensory" Gq, but until more rigid structure-function relationships are established by mutational analyses and heterologous expression, the role of the protein remains obscure.

Localization of the message to the olfactory organ (Figure 11) is consistent with this cDNA encoding the native protein characterized by Fadool *et al.* (1995): any transduction-related protein must be localized to the olfactory organ. Northern blot analysis shows that the Gq message expressed in the olfactory organ is very large compared to the coding sequence. This indicates large 5'- and 3'-UTRs on the mRNA which are over three times the length of the coding region. The significance of this large noncoding region is unknown, but is not unheard of for this family of proteins (e.g.

Figure 11. A Northern blot of nose total RNA (150 μg) probed with a ^{32}P-labeled riboprobe transcribed from a portion of the lobster Gq protein. A single 4.6 kb band (arrow) is seen after a 96 hour exposure.


kb
9.5
7.5
4.4
2.4
1.4

Wilkie *et al.*, 1991). Other tissues were not probed for the expression of this message. Although the question of tissue specificity is an interesting one, it has no bearing on the role of any protein in olfactory transduction. Indeed, each of the principal components of the vertebrate cAMP-mediated olfactory transduction pathway are seen in non-olfactory regions of the nervous system. Nevertheless, the transcript must be localized to ORNs if a link between this message and the native protein is to be established definitively.

This isolated Gq was compared with the one previously implicated in excitatory odor transduction in the spiny lobster. In their study, Fadool and colleagues (1995) found that an antibody against a common sequence of $G\alpha_{q/11}$ recognized a 45 kDa protein in the dendrites of lobster ORNs. This same antibody, when perfused into cultured lobster ORNs, was able to block the inward odor-evoked current, though it had no effect on the outward odor-evoked current. Furthermore, both inward and outward currents were insensitive to the presentation of either pertussis or cholera toxins. Proteins of the Gq family are not sensitive to either pertussis or cholera toxin (Strathmann and Simon, 1990). Together, these data clearly implicated a Gq-like protein in spiny lobster excitatory odor transduction. But although this protein shares immunological and pharmacological properties with known Gq proteins, its membership in the Gq family remained in question. The cDNA isolated from olfactory organ is consistent in primary structure with the Gq-like protein described by Fadool *et al.* (1995). The C-terminal amino acids that served as the epitope for the $G\alpha_{q/11}$ antibody used in that study are precisely conserved in the protein encoded by the cDNA isolated here; this epitope is conserved in most $G\alpha_{q/11}$ proteins, but is noticeably different in two Gq proteins from another arthropod, *Drosophila* (Lee *et al.*, 1990). The computed molecular weight, which is consistent with other Gq proteins, is just slightly smaller than the 45 kDa protein seen by Fadool and colleagues (1995) on Western blots; this discrepancy may be due to lipid modifications of the native protein (Spiegel *et al.*, 1991) or to anomalous migration of the protein in SDS-PAGE. The sequence also reveals a lack of the C-terminal cysteine that

serves as the site of pertussis toxin ribosylation (Simon *et al.*, 1991). These features demonstrate that a Gq protein that could be the one characterized functionally by Fadool and colleagues (1995) is expressed in the nose of the spiny lobster. However, of the several other G protein antibodies screened in that study, one raised against a peptide from a mammalian $G\alpha_o$ could also block the inward odor-evoked current, though this $G\alpha_o$ antibody did not recognize any proteins on Western blots of ORN dendritic membrane. Members of the Gi family are pertussis toxin-sensitive (Simon et al., 1991), further complicating interpretation of the physiological experiment. Note that the epitope of the $G\alpha_o$ antibody, the antigenic peptide LDRIGAADYQ (Goldsmith *et al.*, 1988), is somewhat conserved in the Gq sequence isolated here (Figure 9), suggesting that crossreactivity of the $G\alpha_o$ antibody with the native Gq protein led to the equivocal results seen by Fadool and colleagues (1995). However, no attempt was made to isolate a $G\alpha_o$ cDNA from this tissue, so the possibility that the olfactory organ expresses both types of α-subunit cannot be excluded. To find multiple G proteins involved in the same signalling pathway would be unprecedented in olfaction, but this has been seen in other systems. Some receptors can be coupled to multiple effectors via different G proteins [e.g. human thyrotropin receptors activate both $G\alpha_s$ and $G\alpha_{q/11}$ in intact cells (Allgeier *et al.*, 1994)]. As each ORN was tested with only one of the antibodies, the prospect remains that individual ORNs could express either or both of the α-subunits. The finding by Fadool *et al.* (1995) that the inward and outward currents are mediated by different G proteins is consistent with previous studies that implicated separate second messenger cascades in excitatory and inhibitory odor transduction in this animal (Fadool and Ache, 1992; Michel and Ache, 1994; Boekhoff *et al.*, 1994). If more than two G proteins were to be implicated in one of these pathways, it would imply a complexity of signaling pathways that is not yet understood.

Gq proteins are seen in ORNs of both vertebrates and invertebrates (Abogadie *et al.*, 1995a; Talluri *et al.*, 1995; Dellacorte *et al.*, 1996), including one cloned from the

olfactory organ of the American lobster (Xu *et al.*, 1997). Gq-like immunoreactivity has also been observed in neurons of the vomeronasal organ but was deemed too faint to be of significance for sensory transduction in these cells (Berghard and Buck, 1996). While evidence for a role of the IP_3 pathway in vertebrate olfactory transduction is growing, no role for a Gq has been identified. In fact, Boekhoff and colleagues (1990) have implicated a pertussis toxin-sensitive G protein in IP_3-mediated olfactory transduction. It is possible that in vertebrate ORNs, the IP_3 pathway is coupled to a $G\alpha_o$ or to some member of the Gq family not recognized by antibodies against $G\alpha_q$ or $G\alpha_{11}$ (e.g. $G\alpha_{14}$, $G\alpha_{15}$ or $G\alpha_{16}$).

CHAPTER 4
SUMMARY

Inositol 1,4,5-trisphosphate (IP_3) has been implicated as a second messenger involved in olfactory transduction for a variety of species (review: Breer and Boekhoff, 1992; Ache and Zhainazarov, 1995), though its precise role remains controversial, especially in vertebrates. Several laboratories report that they have failed to identify IP_3-gated conductances in ORNs of amphibians (e.g. Firestein *et al.*, 1991; Nakamura *et al.*, 1994; Kleene *et al.*, 1994). The work of Nakamura and colleagues (1996) may imply that IP_3- and cyclic nucleotide-gated channels (CNGs) are in different cellular compartments, with only the CNG residing in the membrane of ORN cilia. Most recently, gene knockout experiments with the mouse olfactory CNG show that the absence of this channel causes total anosmia in neonates (Brunet *et al.*, 1996). These results contradict those of Restrepo *et al.* (1990), Miyamoto *et al.* (1992) and Lischka *et al.* (1995) who have demonstrated the presence of IP_3-gated conductances in the ORNs of rats and catfish. As has been described extensively in the previous chapters, there is also biochemical evidence for odor-stimulated generation of IP_3 in ORNs, as well as emerging immunological and molecular evidence placing components of an IP_3 signaling pathway in dendrites of these cells. These conflicting data leave the precise role of IP_3 in vertebrate olfactory transduction somewhat ambiguous: IP_3 has been proposed by some to be either a modulatory factor or a necessary cofactor for the better characterized transduction pathway mediated by adenosine 3',5'-cyclic monophosphate (cAMP); others believe it to have a role equivalent, but not identical, to cAMP.

A key to understanding the functional intricacies of the cAMP pathway was the molecular isolation and characterization of components of this signaling cascade in

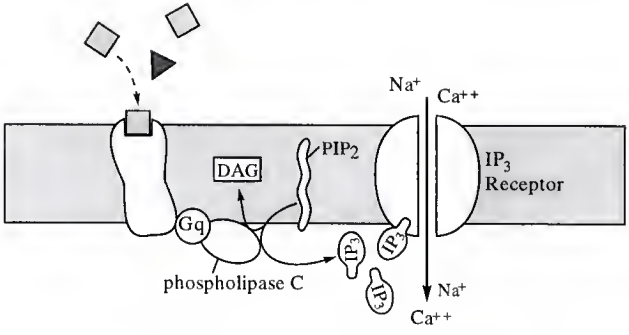
olfactory receptor neurons. This was done, not by random chance, but by taking advantage of perceived similarities of the olfactory cAMP transduction cascade to the cGMP signaling pathway in vertebrate photoreceptors. Homologous (e.g. the olfactory CNG) and analogous (e.g. G_{olf}) proteins were identified by narrowing the search to molecules like those seen to be involved in the much better characterized photoresponse. In this dissertation I have taken a similar tack, using the better characterized IP_3-mediated olfactory transduction pathway in ORNs of the spiny lobster as a source of information about the functioning of IP_3 in olfactory transduction in general.

In the spiny lobster, *Panulirus argus*, electrophysiological studies have implicated an IP_3-gated channel, the IP_3 receptor (IP_3R), as the target of IP_3 generated upon excitatory odor-stimulation. A Gq-type GTP-binding protein α-subunit (Gq protein) has also been implicated in the excitatory odor response, serving to link the activated odor receptor with the enzyme that generates IP_3 (Figure 12). This dissertation describes the molecular cloning of cDNAs for two components of an IP_3 signaling cascade from the olfactory organ (nose) of the spiny lobster: an IP_3 receptor (Chapter 2) and a Gq protein (Chapter 3). These two proteins, both of which have been physiologically implicated in the excitatory odor response in lobsters, share many structural characteristics with those isolated from other species and tissues. The IP_3 receptor cDNA encodes a protein that is approximately 50% identical to many of those isolated from *Drosophila* and several vertebrates. The Gq protein shows an even greater similarity to other Gq proteins (70-80%). The messenger RNAs for these two molecules are both expressed in the olfactory organ, making them candidates for roles in olfactory transduction. The isolation of these two cDNAs is a critical step in our understanding of both the role of IP_3 in olfactory signaling and the complexity of odor transduction in all animals.

Much work remains to be done with both of these molecules to establish that each plays a role in olfactory transduction, rather than elsewhere. Both proteins are quite similar to ones seen in a variety of neural and nonneural cells, so some form of cellular

Figure 12. The proposed lobster excitatory odor transduction pathway, with a Gq protein coupling the odor receptor to a phospholipase C, and IP_3 directly gating a plasma membrane channel.

EXCITATORY
PATHWAY



localization, such as *in situ* hybridization, is necessary to demonstrate that these particular genes are expressed in ORNs (while ORNs are the principal cell type in the olfactory epithelium, other neuronal and nonneuronal cell types are present). The Gq protein has already been shown to be present in the ORN dendrites (Fadool *et al.*, 1995). Two polyclonal antibodies directed against a conserved and a nonconserved region of the IP_3R have been generated, and are currently being characterized. The presence of antigen for both of these antibodies in the ORN dendrites would provide a strong link between the cloned channel and those first characterized by Fadool and Ache (1992). Heterologous expression of both these proteins would permit comparisons to those native proteins previously characterized (Fadool and Ache, 1992; Hatt and Ache, 1994; Fadool *et al.*, 1995). Such experiments would also permit mutational and chimeric studies of these proteins, which might provide insight into numerous structural and functional questions, the most obvious of which is the role for the novel hydrophilic sequence in the modulatory domain of the lobster IP_3R.

Of course, while characterization of these molecules provides great insight into the functional properties of the molecules themselves, the advantage of a comparative approach is the clues it can provide about other systems. If these two molecules are shown to be involved in olfactory transduction in the spiny lobster, the next step would be an attempt to clone and localize equivalent molecules in vertebrate ORNs. Towards this end, a partial cDNA encoding the carboxyl end of a novel IP_3R has been cloned from *Xenopus* olfactory epithelium (Munger, Rust, Ache and Greenberg, unpublished data). Isolation of similar molecules does not guarantee similar roles for the IP_3 pathway in lobsters and vertebrates, but it will simplify the testing of various hypotheses.

While I have concentrated on the IP_3 pathway in ORNs as it relates to primary olfactory transduction (coupling an odor-bound receptor to a change in membrane conductance), activation of this pathway can have additional consequences for the cell and its response to odors. For example, Ca^{2+} entering the cell through the IP_3R, along

with diacylglycerol cogenerated with IP_3 by activated phospholipase C, could activate protein kinase C, which could, in turn, phosphorylate numerous cellular proteins. The mechanisms of olfactory transduction already appear to be subject to complex regulation. Gaseous messengers such as nitric oxide (NO) and carbon monoxide (CO) and the cyclic nucleotide guanosine 3',5'-cyclic monophosphate (cGMP) appear to have modulatory effects on the CNG channel (Breer *et al.*, 1992; Lischka and Schild, 1993; Leinders-Zufall *et al.*, 1995; Zufall, 1996; Broillet and Firestein, 1996). As in most systems, Ca^{2+} seems to have numerous biochemical and physiological effects that may modify the odor response (e.g. Anholt and Rivers, 1990), including playing a large role in the amplification of the initial odor-induced current via the direct gating of a chloride channel (Kleene, 1993; Lowe and Gold, 1993; Kurahashi and Yau, 1994). There is even evidence for a Na^+-gated channel playing a similar amplification role in the lobster (Zhainazarov and Ache, 1995; Zhainazarov *et al.*, 1997). While the existence of these additional transduction mechanisms does not diminish the need to understand the cAMP and IP_3-mediated systems, none of these mechanisms operates in isolation from the others. However, to understand the interactions between these multiple pathways, the components of each pathway must be understood, for each component is a possible site of interaction with another.

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BIOGRAPHICAL SKETCH

Steven Dunn Munger was born on November 26, 1966, in Richmond, Virginia. His parents Peter and Joan Munger moved to Charlottesville, Virginia, 6 months later, taking Steven with them. His only brother, David, was born in 1970. Steven attended Venable and Greenbrier Elementary Schools, Walker Middle School and Charlottesville High School, graduating from the latter in June of 1985. That fall he enrolled at the University of Virginia, where he graduated with a Bachelor of Arts in Biology 4 years later. During his stay at U.Va., Steven worked with Dr. DeForest "Mike" Mellon on a project concerning the projection of olfactory neurons from the nose to the brain. This was his first exposure to olfaction, and served to whet his appetite.

After graduating from U.Va., Steven worked for one year as a technician in the laboratory of Dr. Sally Moody and Dr. Steve Klein. This laboratory focused on development, including development of the nervous system, another influence that would be felt later on.

Steven enrolled as a graduate student at the University of Florida Department of Neuroscience in the fall of 1990, and immediately started to work in the laboratory of Dr. Barry Ache at the Whitney Laboratory. There, after a series of failed projects, he succeeded in isolating two cDNAs from the lobster nose that may play a role in olfactory transduction. While at the Whitney Laboratory, he also collaborated with the laboratory of Dr. Barbara-Anne Battelle on a study characterizing a component of the horseshoe crab visual transduction cascade.

Steven and his new dog Weaver now leave St. Augustine for Baltimore, Maryland, where Steven will take a postdoctoral fellowship with Dr. Randall Reed at the

Howard Hughes Medical Institutes and the Department of Molecular Biology and Genetics, Johns Hopkins University Medical School (Weaver will just play with tennis balls).

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Barry W. Aelie, Chair
Professor of Neuroscience

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and Neuroscience

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Gerard Shaw
Professor of Neuroscience

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Education and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May, 1997

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